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NIST-TRACEABLE NMR METHOD TO DETERMINE QUANTITATIVE WEIGHT PERCENTAGE PURITY OF MUSTARD (HD) FEEDSTOCK SAMPLES

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14. ABSTRACT
A National Institute of Standards (NIST)-traceable method for using nuclear magnetic resonance (NMR) with proton detection is described for determining the weight percent purity of feedstock samples of sulfur mustard (HD). A precision and accuracy test was done. An alternative method for ^{13}C NMR detection is also described.

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PREFACE

The work described in this report was authorized under contract no. W911SR-10-D-0004. This work was started in January 2012 and completed in May 2012.

The use of either trade or manufacturers' names in this report does not constitute an official endorsement of any commercial products. This report may not be cited for purposes of advertisement.

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NIST-TRACEABLE NMR METHOD TO DETERMINE QUANTITATIVE WEIGHT PERCENTAGE PURITY OF MUSTARD (HD) FEEDSTOCK SAMPLES

1. INTRODUCTION

This procedure is based on published Technical Report procedures for using Nuclear Magnetic Resonance (NMR) instruments for determining the purity of CW agent samples.[1,2,3,4,5] Previous National Institute of Standards and Technology (NIST)-traceable methods were described for HN-3[6] and HN-1.[7] The procedure utilizes an internal standard with a known purity to establish an absolute weight percentage for the analyte of interest. Identifying the structures of other components in the mixture is not necessary. All that is necessary is to know the NMR chemical shifts of the major analyte, the internal standard, and the molecular weights. The weight percent calculations are not negatively affected by the presence of unidentified compounds or undetectable components in the sample (for example, inorganic salts, insoluble solids, etc.).

The procedure has been adapted to use a NIST standard material. An internal standard was purchased from Sigma Aldrich that has a NIST-traceable purity. A balance calibrated with NIST traceable weights was also used. These modifications make the method NIST-Traceable. To determine the purity of sulfur mustard (HD), proton NMR was used for detection. Precision and accuracy testing of the method is discussed.

A confirmation NMR method is also discussed that uses ^{13}C NMR. This method can also be NIST traceable. The method was not tested using a Precision and Accuracy test due to limited amounts of time and CW agent.

2. PROCEDURE

2.1 Supplies

The following supplies can be used for the procedure. Equivalent supplies may be available from other vendors.

The NIST-traceable internal standard was 1,2,4,5-tetramethylbenzene, purchased from Fluka (Sigma Aldrich), Part Number 74658-5G, CAS No. 95-93-2, as a TraceCERT® certified reference material (CRM) standard for quantitative NMR. For ^{13}C spectra (discussed further in Section 3), an internal standard of 1,1,1,2-tetrachloroethane is used, purchased from Sigma Aldrich, Part Number T7209-25G, CAS No. 630-20-6, ReagentPlus® 99% purity.

The following supplies were purchased from Wilmad (1172 NW Boulevard Vineland, NJ 08360, phone 800-220-5171, http://www.wilmad-labglass.com/ordering/index.jsp):

Item	Part Number
5 mm dia. 8" long NMR tube	WG-1000-8-50
Teflon inserts	6005
pasteur pipets, 9"	C-7095B-9

The following supplies were purchased from Sigma Aldrich (http://www.sigmaaldrich.com/chemistry.html):

Item	Part Number
chloroform, 99.9% D	23,689-6

For the Precision and Accuracy testing, a JEOL ECS-400 Nuclear Magnetic Resonance spectrometer with a 400 MHz (9.8 T) superconducting magnet and 5 mm liquid analysis probe was used. A Sartorius Cubis balance (Model MSA6.6S-000-DM, precision 1 µg) was used after installation in a fume hood and calibration using NIST-traceable weights. NMR systems and balances from other vendors should give comparable results, if the operators have the appropriate training.

Other common laboratory equipment is used, including a vortex mixer, spatulas, and volumetric pipets. This equipment is not critical to the accurate performance of the method.

2.2 Sample Preparation

This procedure was performed under proper engineering controls, in accordance with surety and safety regulations, equipment validations, and SOPs approved by the ECBC Safety and Health Office. The balance must be calibrated using NIST-traceable weights.

a. Tare a screw-cap vial with cap on the balance. Transfer 10-20 mg of neat internal standard, the NIST traceable 1,2,4,5-tetramethylbenzene, into the vial. This compound is a solid material, so it is transferred with a clean spatula. Replace the cap and determine the weight of the internal standard to an accuracy of 0.01 mg. Tare the balance after recording the weight.
b. Add 5-35 mg of feedstock agent sample to the vial. The liquid agent can be measured with a pipet (4 to 30 µl of liquid). (A precision and accuracy test of this method has been done over this range of agent amounts, see Appendix I.) Record the weight to an

accuracy of 0.01 mg in a laboratory notebook. Appropriate agent accountability documentation is used to record the consumption.

c. Add 0.4 ml of reagent-grade deuterated chloroform ($CDCl_3$).
d. Vortex or mix the sample for at least 15 s to dissolve both compounds in the solvent.
e. Transfer the solution into a PTFE NMR tube insert. (Optional: A glass 4mm insert tube may be used, and flame sealed, if desired)
f. Place the insert into a 5 mm glass NMR tube and push it to the bottom of the tube. Cap the insert with a PTFE stopper. Cap the NMR tube with a cap, or flame seal the outer tube without damaging the insert.

2.3 Obtaining the NMR spectrum

Operators of the NMR must have sufficient training to understand the general operational principles and to use the instrument computer control to perform the required tasks. To validate the NMR is functioning correctly, a manufacturer sample such as 0.01% ethylbenzene in deuterated acetone can be analyzed to check the signal response. The analysis of this sample can be done periodically as part of the instrument QC validation. Detailed QC specifications are not included in this method.

a. Place the NMR tube into the spinner using a depth gauge to orient the tube at the correct depth relative to the detection coils. Activate the lift air supply, place the sample on top of the magnet, and deactivate the lift air supply to lower the sample into the magnet bore. (Note: The doubly-contained NMR tube that contains agent will be outside of engineering controls.)
b. Lock the instrument on the deuterium signal from the $CDCl_3$.
c. Shim the magnet to maximize the lock signal.
d. Tune and match the probe. (Tune for the optimal signal response, and impedance match the probe to the sample. On some instruments, this operation is done automatically by the instrument software and autotune equipment. On older instruments, it must be done using manual adjustments on the NMR probe.)
e. OPTIONAL: Determine the T_1 relaxation time of the analytes in the sample solution. Use the instrument console to load the data file or instrument parameters for an inversion recovery experiment for proton detection. Perform the experiment with at least six delay times. Process the data to plot the recovery curve for each analyte peak, and determine the T_1 relaxation time from the data plot. Identify the longest T_1 value for all the peaks. The relaxation delay time for the quantitative purity measurement is calculated to be at least 10 times the longest relaxation time. This procedure to determine the T_1 relaxation time should be done if there is an inconsistency in the purity determination, if a new instrument is being used, or if it is necessary to minimize the experiment acquisition time.

f. Load instrument parameters to acquire a 1D proton spectrum. If the T_1 relaxation time is not determined (i.e., step e is not performed), then set the relaxation time to 40 s. (This is typically 20 times longer than the longest T_1 in the solvent.) Do not use Nuclear Overhauser Enhancement (NOE), decoupling, or water peak suppression pulse sequences.

g. Open a new data file on the NMR computer with a unique filename, the sample information, and notebook reference. Load the parameters for proton acquisition. The following parameters are used. (Actual parameter names will vary depending on the make and model of the NMR and can be found in the NMR documentation.):

    Relaxation time: 40 s or as determined in step e.
    Excite pulse: 90º pulse (Determining the time and amplitude for this pulse that corresponds to a 90° proton excitation should be found in the NMR instrument documentation.)
    Number of data points: 64K
    Number of scans: 16
    Sweep width: 15 ppm
    Center frequency: 5 ppm
    Decoupling: off
    NOE: off
    Automatic gain determination: on

h. Acquire data.

i. A total of seven or more replicate runs are acquired for statistical determination of the NMR variability, signal to noise ratio, and integration errors. Several samples can be prepared by weight to determine the weighing statistical errors (see Appendix I).

2.4 Data Processing

a. Apply a window function (exponential multiplication). This may be done using a line broadening parameter in the range of 0.5 to 2 Hz, which can be adjusted to enhance the signal to noise ratio. A larger line broadening produces wider peaks, which can degrade the resolution between peaks. The same value of line broadening must be used for all the data files for the repeat runs.

b. Fourier transform (FFT) to convert data from time to frequency domain and to produce the NMR spectrum. A sample spectrum is shown in Figure 1.

c. Phase all peaks in the spectrum and correct the baseline if necessary.

d. If necessary for reporting, reference the chemical shift against the internal standard.

e. Integrate the relevant peaks in the spectrum to obtain the areas. A sample integrated spectrum is shown in Figure 2 with an expanded y-scale. Some data systems will perform automatic integration of peaks. It is necessary for the operator to examine the

integration to make sure that the correct parts of the peak are included in the integration. If the automatic integration is incorrect, the spectrum can be manually integrated. In particular, Figure 2 shows that each peak has two ^{13}C satellite peaks on each side of the main peak. These peaks are produced by molecules that have a natural abundance of ^{13}C isotopes, and they each represent 0.55% of the center peak. The satellite peaks should be included in the integration of the central peak. (If the magnet is not well shimmed, the satellite peaks may not be resolved.)

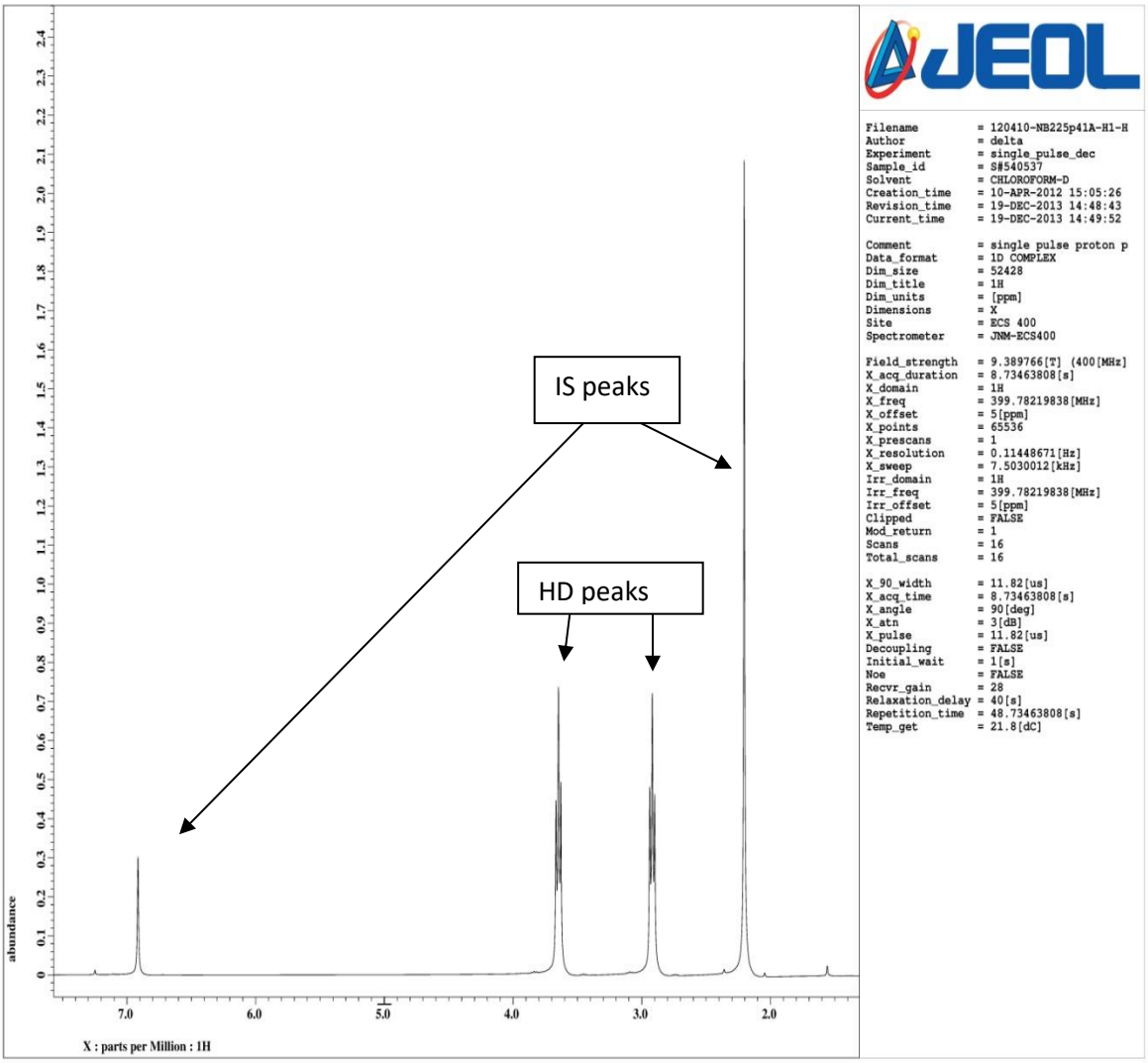

FIGURE 1: Proton NMR spectrum of HD agent and the internal standard 1,2,4,5-tetramethylbenzene.

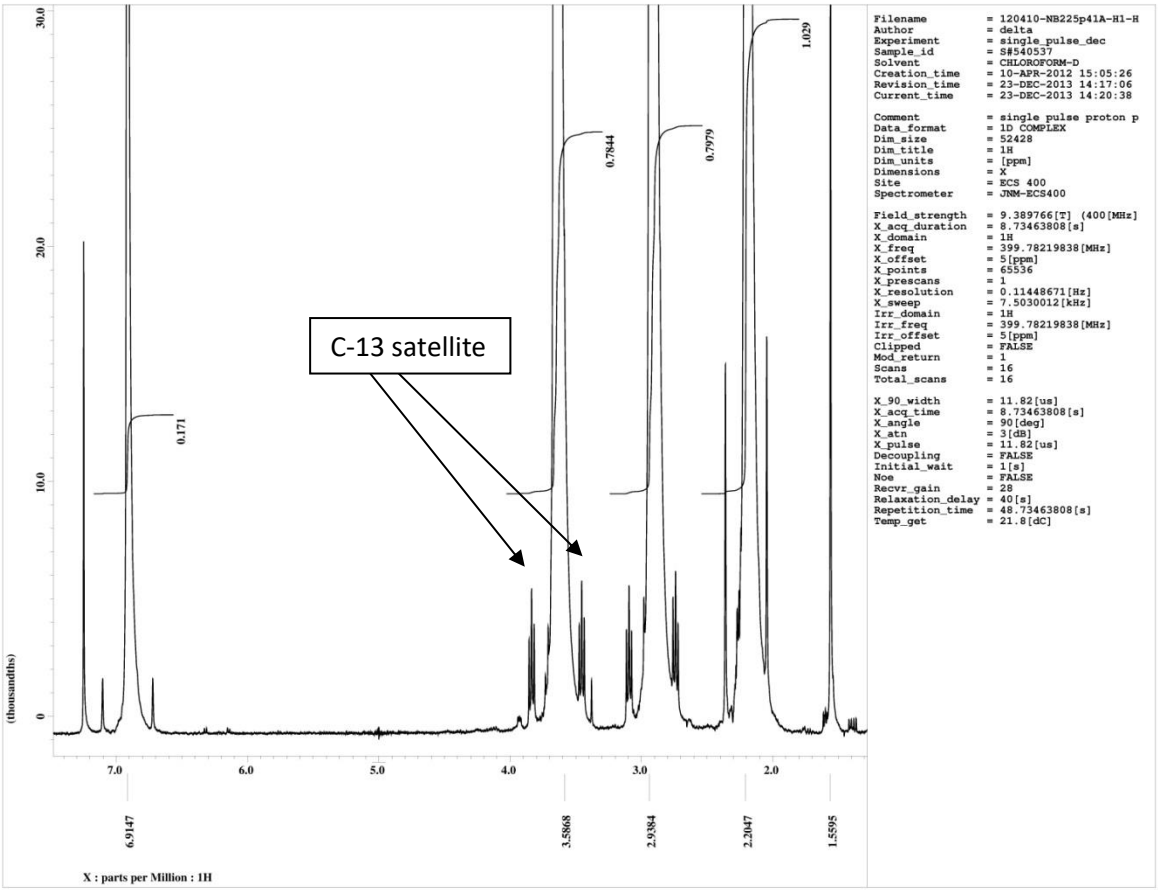

FIGURE 2: Plot showing the spectrum in Figure 1 with an expanded y-scale. The small ^{13}C satellite peaks next to the central peaks are shown. Integrals are shown.

3.5 Purity Calculation

The weight percent of the analyte (Wt% A) in the sample is calculated using the following formula, where analyte A is the HD, and IS is the internal standard:

$$\text{Wt\% A} = \frac{\text{Area under A peak}}{\text{Area under IS peak}} \times \frac{\text{MW of A}}{\text{MW of IS}} \times \frac{\text{Weight IS}}{\text{Weight A}} \times \frac{\text{No. identical H(IS)}}{\text{No. H (A)}} \times (\text{Pur IS})\%$$

Area under A peak = total sum of the area of the triplet peaks and the ^{13}C satellite peaks that are associated with them;
Area under IS peak = total area of the singlet peak at 2.2 ppm and the ^{13}C satellite peaks;
MW of A = average molecular weight of HD, which is 159.08 D;
MW of IS= average molecular weight of the internal standard, which is 134.22 D;

Weight IS=balance recorded weight of internal standard in the vial;
Weight A=balance recorded weight of feedstock HD sample in the vial;
No. identical H (IS)=the number of identical protons in the internal standard, which is 12;
No. H (A)=the number of protons in the integrated peaks of the analyte,
Pur IS = the purity of the internal standard is taken from the documentation from the NIST traceable internal standard.

Two alternatives are possible for integrating the HD peaks. If the total area of both triplets at 2.9 and 3.6 ppm are used, then the total number of protons No. H (A) is 8. When only one triplet is used, usually at 3.6 ppm, then No. H (A) is 4. The reason for using only one triplet is that when the sample contains a significant amount of the impurity dithiane, the NMR peaks for that compound may overlap with the triplet at 2.9 ppm, and cause that triplet to have an area that is too large. There are other impurities that contain C-S bonds that may also have a chemical shift near the 2.9 ppm triplet. This can result in an inaccurate purity determination.

If the analytical statistical accuracy is reported, the calculated weight percentages for each replicate run can be averaged to find a mean (average) and standard deviation. For seven replicates, the mean ± 2 × (standard deviation) provides the 95% confidence range.

3. ALTERNATE METHOD FOR PURITY CONFIRMATION USING ^{13}C SPECTRA

As an alternative method for confirming the results of the purity determination method, the purity can also be found using a ^{13}C spectrum. The advantage of this approach is to provide higher spectral resolution, so that impurities can be more easily identified in the spectrum by resolving them from the HD peaks. The disadvantage is that the ^{13}C spectrum is less sensitive. This is because ^{13}C is only present as 1.1% of all the carbon atoms. As a result, longer data acquisition times are needed to obtain an adequate signal to noise ratio, and the precision of the result is less.

The sample can be prepared following the same procedure as in Section 2. A modification is to use a larger amount of HD and internal standard to compensate for the lower sensitivity. Quantities of up to 200 mg of HD and internal standard can be used in Step b of Section 2.2.

The following NMR instrument parameters can be used, substituting for Step g of Section 2.3:

Load the parameters for 1D ^{13}C acquisition.

Relaxation time: 30 to 60 s or as determined in step e.
Excite pulse: 90º pulse (determining the time for this pulse that corresponds to a 90° pulse should be found in the NMR instrument documentation)
Number of data points: 64K
Number of scans: 128 or more
Sweep width: 400 ppm

Center frequency: 100 ppm
Proton Decoupling: on
NOE: off
Automatic gain determination: on

There are several main modifications for the instrument conditions. First, the proton decoupling is turned on, which increases the sensitivity and decreases the splitting and peak widths. However, this condition causes some distortion of the signal responses for different peaks. Since the decoupling transfers signal intensity to the carbon atom, it is observed that CH_3 groups have the highest relative signal intensity, and others follow in the order of $CH_3 > CH_2 > CH > C$. In order to obtain accurate quantitation for the CH_2 groups in HD, it is necessary to have an internal standard that also has CH_2 groups. A convenient internal standard is 1,1,1,2-tetrachloroethane, purchased from Sigma Aldrich, Part Number T7209-25G, CAS No. 630-20-6, ReagentPlus® 99% purity. However, this standard is not a TraceCERT standard, so in order for the method to be NIST traceable, the standard has to be treated as a secondary standard relative to 1,2,4,5-tetramethylbenzene (or another NIST standard). The purity of the standards can be compared using proton NMR. In practice, the purity that is determined by ^{13}C NMR has lower precision than that for proton NMR, so the uncertainty in the purity of the internal standard is not usually a large contribution to the overall uncertainty.

The second modification is to the relaxation delay time. As discussed in Step e of Section 2.3, the T_1 delay time can be obtained for the peaks in the ^{13}C spectrum in the same way as for a proton spectrum, using an inversion recovery experiment. The T_1 delay times are typically longer for ^{13}C spectra than for proton spectra, so the relaxation delay should be the same or longer for the ^{13}C spectral parameters. But the delay is changed from 40 s for proton to 30 to 60 s for ^{13}C for one acquisition, so that more signal averaging can be done or more repetitions can be done. The number of scans increases from 16 for proton to 128 (or more) for ^{13}C. Smaller values for the relaxation delay can lead to some systematic error of the relative peak areas, but the error is typically less than the random error that is produced by the lower signal-to-noise ratio of the spectra due to the lower signal strength. The signal to noise ratio for a typical spectrum should be at least 50. The measurement of a T_1 value is discussed in Appendix II.

The spectral sweep width of 400 ppm and center frequency of 100 ppm are typical default values for ^{13}C spectra, since the spectra cover a wider range of frequencies than the proton spectra cover. A typical spectrum is shown in Figure 3.

Using these conditions, the purity can be calculated using the same method as described in Section 2.5. A P&A study has not been done on the ^{13}C purity method. A study to optimize the parameters such as the relaxation time and number of scans will be time consuming. Typical testing results are given in Appendix II.

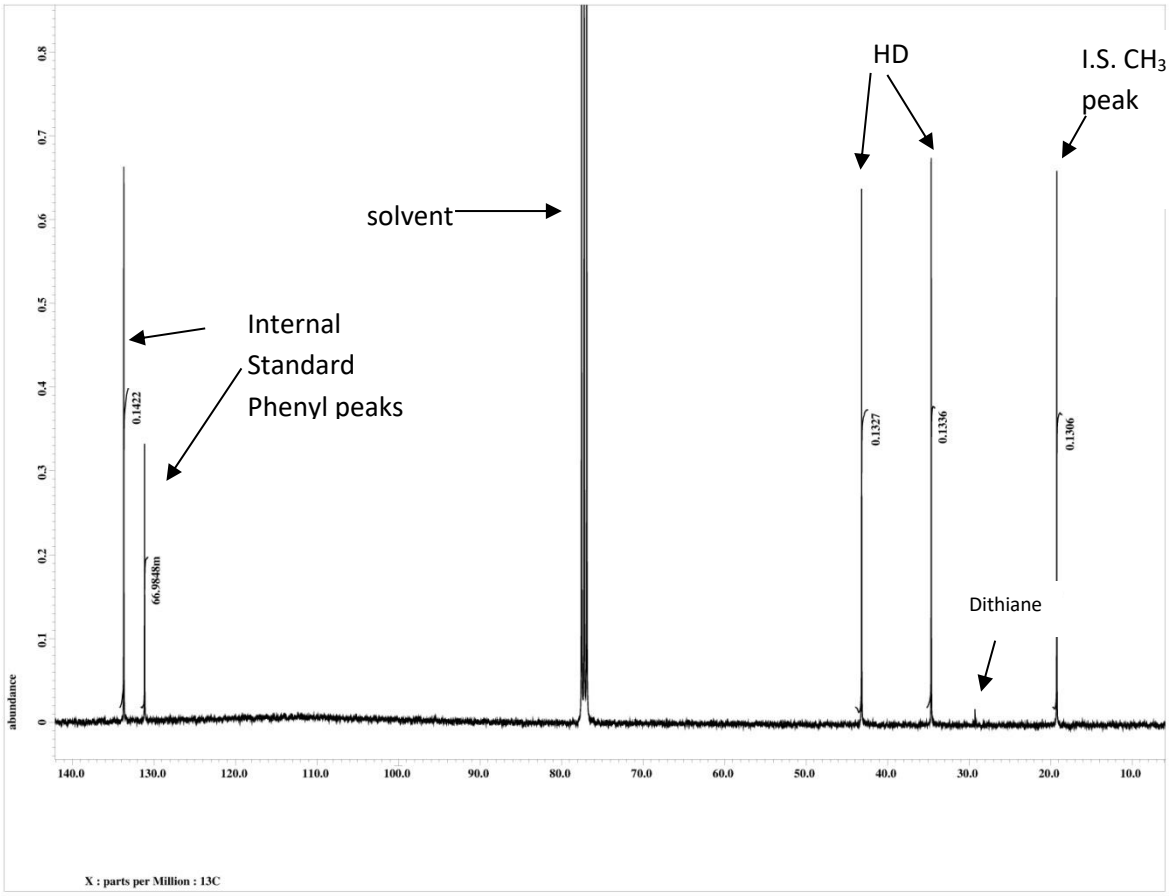

FIGURE 3: Plot showing a ^{13}C spectrum of the same sample used for the proton spectrum in Figure 1 for HD with an internal standard of 1,2,4,5-tetramethylbenzene, in $CDCl_3$ solution. Integrals are shown.

4. CONCLUSIONS

By using the NIST-traceable internal standard, and the balance that is calibrated with NIST-traceable weights, the purity of the CW agent feedstock HD is determined using a NIST-Traceable method with proton NMR spectra. A confirmation method using ^{13}C NMR spectra uses a secondary standard that must be referenced to a NIST-traceable standard, and it has lower precision, but it is capable of resolving and identifying some possible contaminants in the HD.

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APPENDIX I: Precision and Accuracy Testing Data for the NIST-Traceable HD Method using Proton NMR

I-1. APPROACH

The HD purity determination method was validated using a variation of the protocol used in a Class I Precision and Accuracy (P&A) test. This kind of test is typically used for validation of air monitoring methods. The requirements are not exactly applicable to an NMR purity determination test, so it was modified as needed.

A four-day test was used. On each day of the test, 10 samples and two blanks were prepared. The 10 samples were prepared with amounts of HD of 0.2Z, 0.5Z, 0.8Z, 1.0Z, and 1.5Z, each sample in duplicate, where Z = 25 mg of HD. As a result, the purity method was validated for a quantity of agent from 5 mg to 37.5 mg.

This testing was not in strict accordance with a normal P&A test. First, NMR is not a trace detection method, and the purpose of the method is not to detect low amounts of agent for safety purposes, as it is for air-monitoring applications. For a typical Class I P&A, the amount of agent is measured in nanograms, usually dictated by the need to detect mandatory exposure limits. The NMR method is measured in milligrams, and the Z level is arbitrarily based on detection limits of the current instrument.

The data from a P&A test is typically processed using a program called Certify (latest version is version 6.0). Certify contains statistical criteria for the acceptance of data or the test method within acceptable measurement limits. Certify does not apply to the NMR purity determination very well, however. The target Z levels (where Z is the target amount) are set in the program to be the same for all replicates from the four-day test. The approximate target level is measured using an adjustable pipet, chosen according to the target levels. For the NMR purity method, the actual amount of agent is determined by the weight of the agent taken from the NIST-traceable balance. The accurate amount is different and more accurately known for each sample of the 4-day test, even if the nominal target Z is the same. The accurate weight cannot be entered into the Certify program as an x-coordinate, only the target Z level.

The P&A test was performed on a JEOL ECS-400 Nuclear Magnetic Resonance Spectrometer, installed in July 2011 to meet the manufacturer's specifications. Data was collected and processed by operators who have received software and hardware training from JEOL representatives.

Weighing was done using a Sartorius Cubis balance, barcode 9804. The balance was calibrated by the ECBC Calibration Team on 30 Nov 11 (expires 29 Nov 12) by Reese (W959QC), identification number 27102674.

The internal standard was 1,2,4,5-tetramethylbenzene, purchased from Fluka (Sigma Aldrich), Part Number 74658-5G, CAS No. 95-93-2, as a TraceCERT® certified reference material (CRM) standard for quantitative NMR. The lot number is #BCBC1486, Pcode number 100975857, expiration June 2012. The standard was received with a certification of analysis of 99.95% (g/g), and uncertainty of 0.08% (g/g).

The T_1 for the solutions (see Section 2.3 step e) was not determined, and 40 s was used as the NMR relaxation delay time.

I-2. RESULTS

Tables I-1 to I-4 show the data sets collected on each day of the four-day P&A test. Figure I-1 shows the data plotted together with the regression lines and correlation coefficients.

Table I-1: Data from Day 1.

Area of Analyte (agent)	Area of Standard	Wt. Of Standard	Sample Weight	Z (wt agent/ 25 mg)	Found Z
212.53	79.3407	7.78	37.12	1.4848	1.4813
186.9916	75.181	8.54	37.62	1.5048	1.5097
137.7546	99.0741	10.43	25.08	1.0032	1.0308
106.8118	50.6484	6.77	25.2	1.008	1.0148
90.9133	62.4321	7.68	19.77	0.7908	0.7949
88.6052	73.2226	9.34	20.09	0.8036	0.8033
76.7406	100.5017	9.63	12.59	0.5036	0.5226
64.8385	104.1854	11.44	12.63	0.5052	0.5060
28.2555	90.1932	8.71	4.85	0.194	0.1939
20.4064	77.7134	10.15	4.92	0.1968	0.1894
0	107.5583	12.9	0	0	0.0000
0	108.4889	13.82	0	0	0.0000
correlation coefficient					0.99975

Table I-2: Data from Day 2.

Area of Analyte (agent)	Area of Standard	Wt. Of Standard	Sample Weight	Z (wt agent/ 25 mg)	Found Z
196.2199	92.8684	9.9	37.14	1.4856	1.4868
156.5142	58.1506	7.79	37.2	1.488	1.4903
113.7556	101.0534	12.41	24.91	0.9964	0.9929
138.1683	119.3806	12.04	24.81	0.9924	0.9905
154.1801	110.0161	8.14	20.2	0.808	0.8108
85.3597	73.9417	9.93	20.4	0.816	0.8148
53.4678	46.9613	6.05	12.38	0.4952	0.4896
53.7328	71.7037	9.51	12.59	0.5036	0.5065
22.6562	62.1186	7.93	5.09	0.2036	0.2056
23.6294	57.1665	7.11	5.21	0.2084	0.2089
0	128.4253	16.3	0	0	0.0000
0	72.0354	8.93	0	0	0.0000
correlation coefficient					0.99998

Table I-3: Data from Day 3.

Area of Analyte (agent)	Area of Standard	Wt. Of Standard	Sample Weight	Z (wt agent/ 25 mg)	Found Z
262.8227	143.566	11.78	38.31	1.5324	1.5328
235.8428	137.2415	12.53	38.27	1.5308	1.5305
103.4077	149.4092	20.3	25.97	1.0388	0.9986
103.8643	104.4185	14.14	25.02	1.0008	0.9997
86.0112	94.7035	12.38	19.99	0.7996	0.7992
84.8575	68.6522	9.13	19.97	0.7988	0.8021
53.3865	99.2361	13.04	12.42	0.4968	0.4986
63.818	143.9302	15.9	12.52	0.5008	0.5011
22.4863	91.3723	10.93	4.79	0.1916	0.1912
29.3519	111.2776	10.92	5.04	0.2016	0.2047
0	88.852	11.5	0	0	0.0000
0	82.3869	10.22	0	0	0.0000
correlation coefficient					0.99964

Table I-4: Data from Day 4.

Area of Analyte (agent)	Area of Standard (IS)	Wt. Of Standard	Sample Weight	Z (wt agent/ 25 mg)	Found Z
229.104	150.1219	13.83	37.54	1.5016	1.5002
155.3662	90.7756	12.19	37.07	1.4828	1.4829
107.78	102.641	13.92	25.69	1.0276	1.0389
104.9311	93.4481	12.63	25.1	1.004	1.0080
86.5421	110.0473	14.51	20.27	0.8108	0.8111
85.1803	78.368	10.5	20.26	0.8104	0.8112
54.8305	106.7944	14.32	13.1	0.524	0.5226
53.6906	93.0464	12.32	12.63	0.5052	0.5053
22.0786	99.6339	13.02	5.1	0.204	0.2051
21.8828	72.4093	9.34	5.03	0.2012	0.2006
0	113.2214	9.3	0	0	0.0000
0	106.4235	9.53	0	0	0.0000
correlation coefficient					0.99997

Correlation coefficients for all the days between the target Z (as a weight) and the found Z are >0.999.

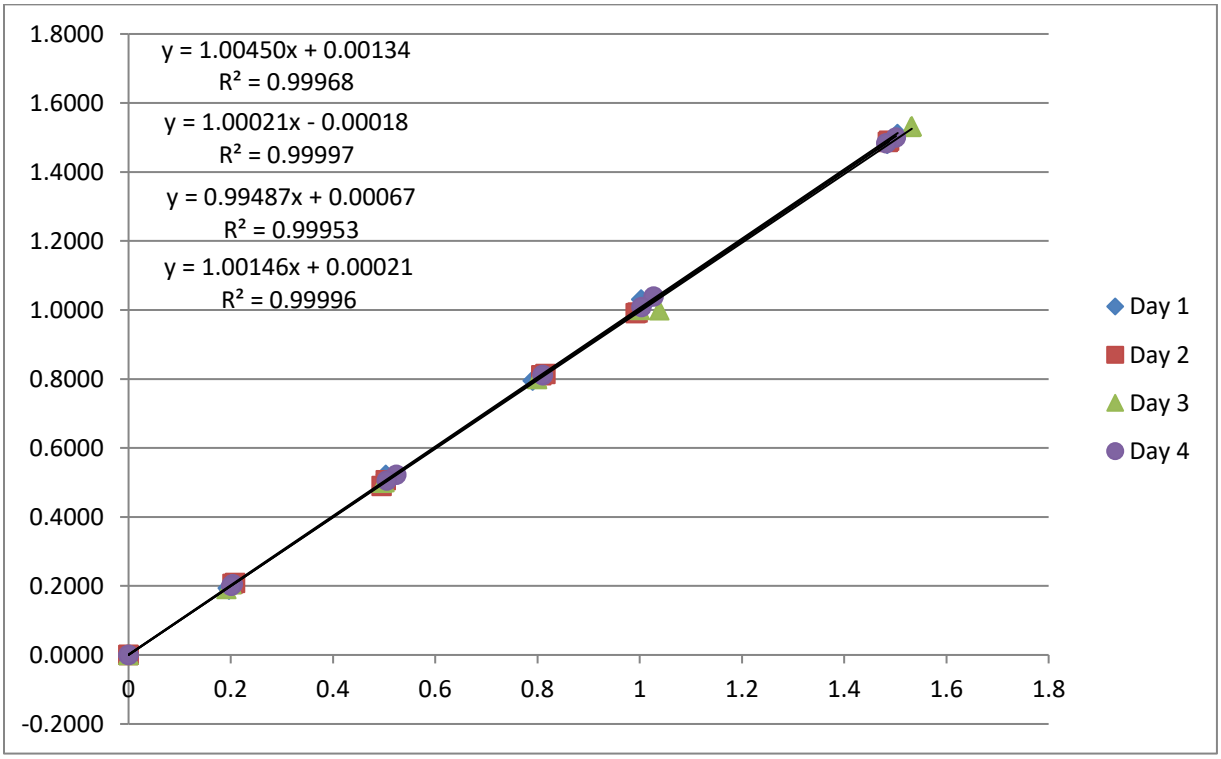


Figure I-1: Plot of the data from four days, Found Z vs. Target Z.

To do the calculation for the Found Z, the formula from Section 2.5 was used, except it was normalized to 1Z = 25 mg instead of using the actual Weight A. Using this method, the purity of the HD sample can be determined from the slopes of the curves from Figure I-1. Averaging all four slopes gives an average purity of 100.03 wt%.

The typical way to determine the purity with this method, without an entire P&A study, is simply to calculate purity for each run using the formula in Section 2.5. Table I-5 shows the calculations for Day 1 data, excluding the blank runs. The resulting average purity is 100.88 wt%, with a standard deviation of 1.4%. The 95% confidence limit is 2.81%.

Table I-5: Data from Day 1, used to calculate purity for each run.

Area of Analyte (agent)	Area of Standard	Wt. Of Standard	Sample Weight	Weight %
212.53	79.3407	7.78	37.12	99.76
186.9916	75.181	8.54	37.62	100.33
137.7546	99.0741	10.43	25.08	102.75
106.8118	50.6484	6.77	25.2	100.67
90.9133	62.4321	7.68	19.77	100.52
88.6052	73.2226	9.34	20.09	99.97
76.7406	100.5017	9.63	12.59	103.78
64.8385	104.1854	11.44	12.63	100.17
28.2555	90.1932	8.71	4.85	99.97
20.4064	77.7134	10.15	4.92	96.26
Average				100.88
Standard Deviation				1.41
Confidence Limits				2.81

For these purity results, the purity value is slightly above 100 wt.%, although it is within the error limits of 100 wt.%. Alternately, the purity can be calculated by using only one triplet peak in the NMR spectrum at 3.65 ppm. It was observed that the area for this peak is systematically lower than the area for the triplet at 2.9 ppm. By using only one triplet, the areas and the calculated purities are shown in Table I-6. The purity value decreases slightly to 99.6 wt.%, which is under 100 wt.%. It is possible that there is a small impurity peak hidden under the 2.9 ppm triplet, which causes the result to be slightly too high, compared to the previously discussed method.

Table I-6: Data from Day 1, but calculating the purity with only the NMR triplet at 3.65 ppm.

Area of Analyte (agent)	Area of Standard	Wt. Of Standard	Sample Weight	Weight %
104.8664	79.3407	7.78	37.12	98.45
92.2222	75.181	8.54	37.62	98.96
68.5251	99.0741	10.43	25.08	102.22
52.7531	50.6484	6.77	25.2	99.44
44.8645	62.4321	7.68	19.77	99.21
43.6973	73.2226	9.34	20.09	98.60
37.8331	100.5017	9.63	12.59	102.33
31.9362	104.1854	11.44	12.63	98.67
13.9137	90.1932	8.71	4.85	98.46
10.0149	77.7134	10.15	4.92	94.48
Average				99.59
Standard Deviation				1.56
Confidence Limits				3.11

To minimize the amount of sample preparation, it is possible to prepare only one sample and rerun it multiple times. This approach minimizes the hazard from handling neat agent and minimizes the consumption of agent and generation of waste. However, the repetitions include only the error that is generated by the NMR data acquisition and integration, and not errors from weighing and sample preparation. Table I-7 shows data from repeated runs of the 1Z sample from Day 1. The error shown by the standard deviation is slightly smaller.

Table I-7: Data from repeated runs of one (1 Z) prepared sample.

Area of Analyte (agent)	Area of Standard	Wt. Of Standard	Sample Weight	Weight %
137.7546	99.0741	10.43	25.08	102.75
139.8176	103.3583	10.43	25.08	99.96
139.9571	103.471	10.43	25.08	99.96
140.2784	103.5179	10.43	25.08	100.14
140.1195	103.4935	10.43	25.08	100.05
139.7688	103.32	10.43	25.08	99.97
140.1056	103.5139	10.43	25.08	100.02
Average				100.41
Standard Deviation				1.03
Confidence Limits				2.07

I-3 Certify results

The results were analyzed by the program Certify 6.0 used for P&A data analysis. The screens that were generated by the program are shown in Figures I-2 to I-5. Parameters that are calculated by the program are shown on the screens.

The data that is obtained from this test is ±3.1%, which passes the Certify pass/fail criteria of ±25%. This is much less accurate than the target accuracy of a purity determination. But because of the way the data is entered, Certify is effectively testing the accuracy of the pipetting, or the correspondence of the target Z with the weight. As shown in Figure I-5, there is no scatter in the x-coordinate. The accuracy of the weighing and NMR determination is better than the accuracy of the pipeting. The actual accuracy of the data from weighing and NMR determination is better than the Certify calculations suggest, so using Certify to quantify the P&A results in this case does not accurately indicate the method performance. As a result, a better way to judge the results is in terms of standard deviations and correlation coefficients of the data.

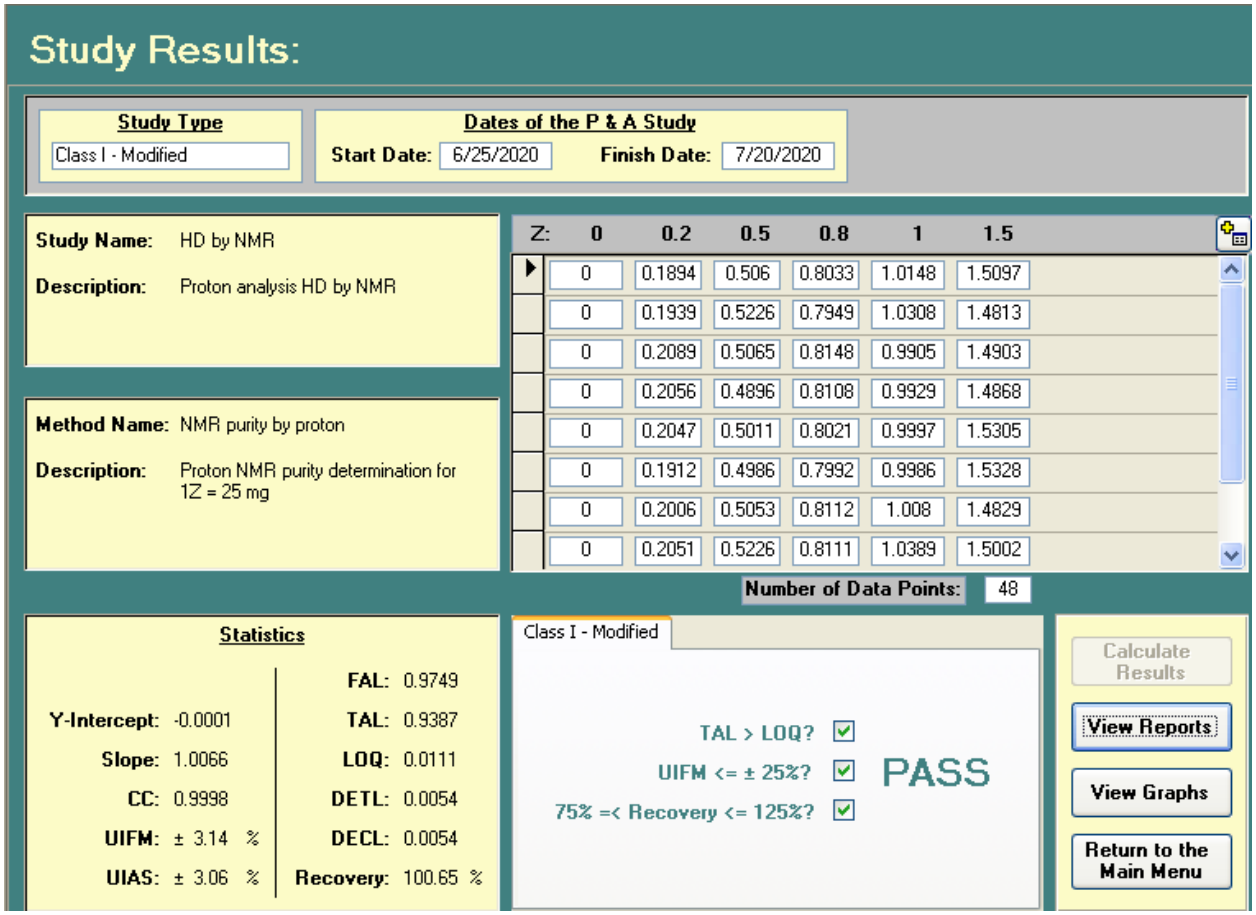
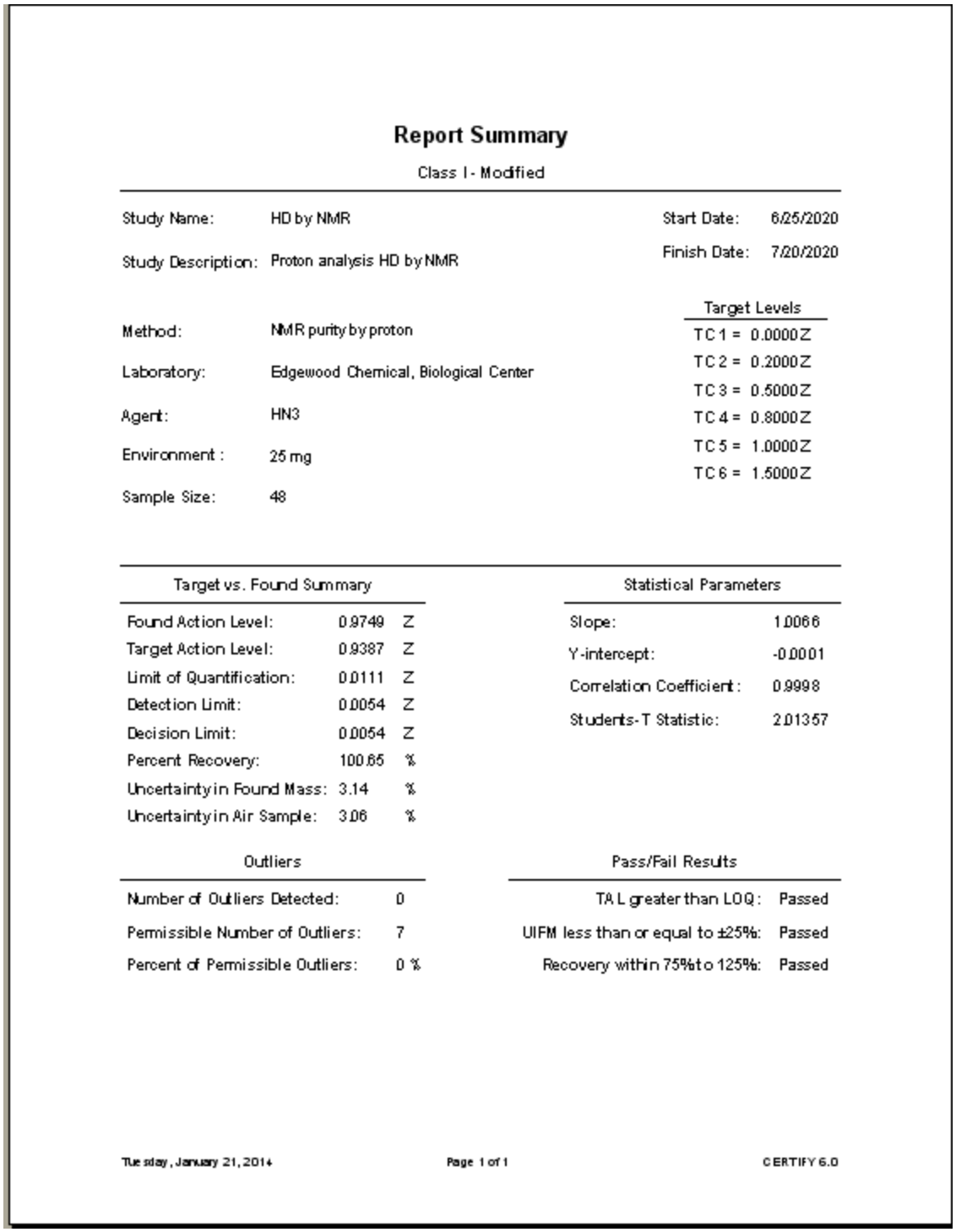


Figure I-2: Certify results page for the four-day P&A study.



Report Summary

Class I - Modified

Study Name:	HD by NMR	Start Date:	6/25/2020
Study Description:	Proton analysis HD by NMR	Finish Date:	7/20/2020
Method:	NMR purity by proton		
Laboratory:	Edgewood Chemical, Biological Center		
Agent:	HN3		
Environment :	25 mg		
Sample Size:	48		

Target Levels

TC 1 = 0.0000 Z
TC 2 = 0.2000 Z
TC 3 = 0.5000 Z
TC 4 = 0.8000 Z
TC 5 = 1.0000 Z
TC 6 = 1.5000 Z

Target vs. Found Summary

Found Action Level:	0.9749	Z
Target Action Level:	0.9387	Z
Limit of Quantification:	0.0111	Z
Detection Limit:	0.0054	Z
Decision Limit:	0.0054	Z
Percent Recovery:	100.65	%
Uncertainty in Found Mass:	3.14	%
Uncertainty in Air Sample:	3.06	%

Statistical Parameters

Slope:	1.0066
Y-intercept:	-0.0001
Correlation Coefficient:	0.9998
Students-T Statistic:	2.01357

Outliers

Number of Outliers Detected:	0
Permissible Number of Outliers:	7
Percent of Permissible Outliers:	0 %

Pass/Fail Results

TAL greater than LOQ:	Passed
UIFM less than or equal to ±25%:	Passed
Recovery within 75% to 125%:	Passed

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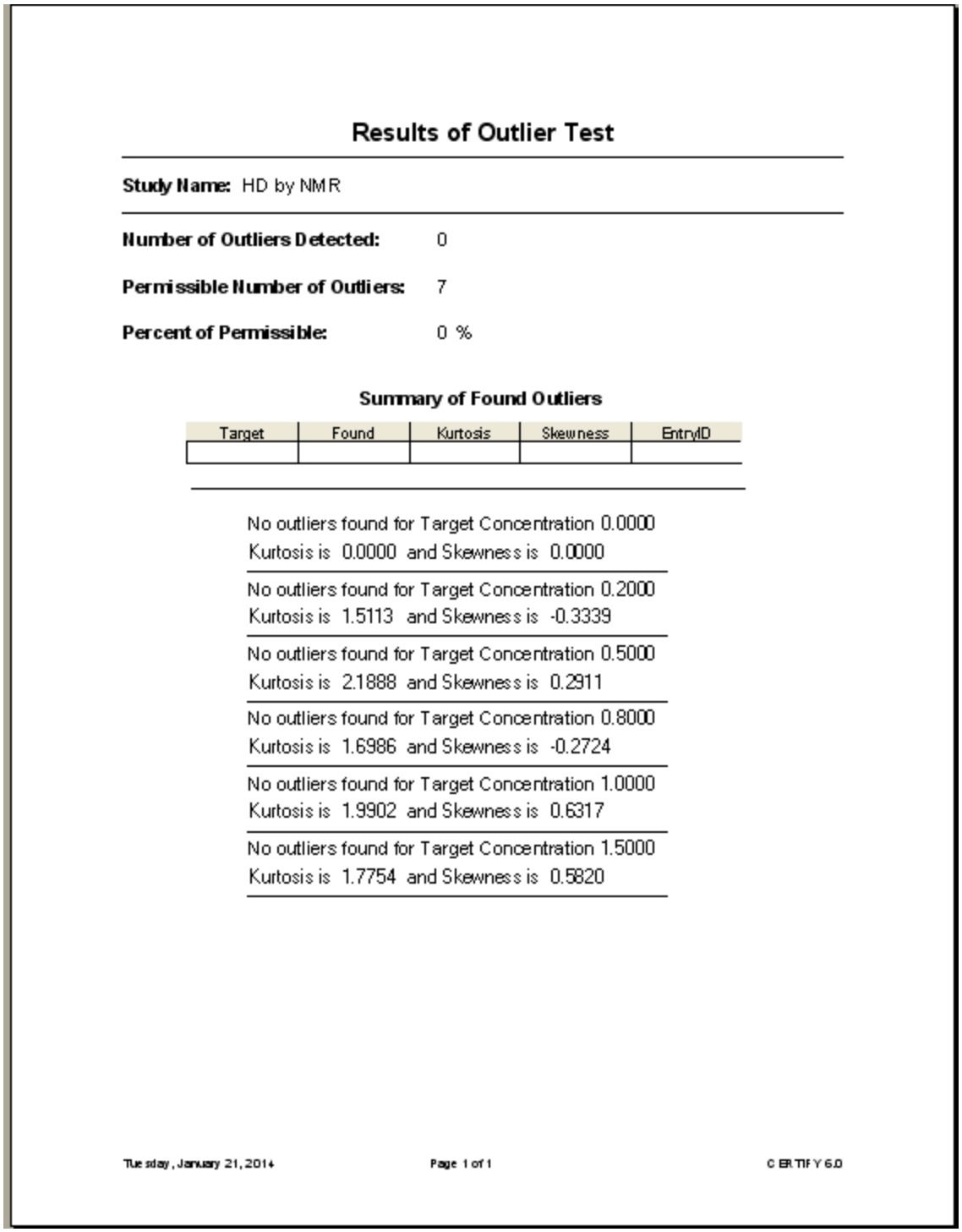
Figure I-3: Certify report summary.



Results of Outlier Test

Study Name: HD by NMR

Number of Outliers Detected: 0

Permissible Number of Outliers: 7

Percent of Permissible: 0 %

Summary of Found Outliers

Target	Found	Kurtosis	Skewness	EntryID

No outliers found for Target Concentration 0.0000
Kurtosis is 0.0000 and Skewness is 0.0000

No outliers found for Target Concentration 0.2000
Kurtosis is 1.5113 and Skewness is -0.3339

No outliers found for Target Concentration 0.5000
Kurtosis is 2.1888 and Skewness is 0.2911

No outliers found for Target Concentration 0.8000
Kurtosis is 1.6986 and Skewness is -0.2724

No outliers found for Target Concentration 1.0000
Kurtosis is 1.9902 and Skewness is 0.6317

No outliers found for Target Concentration 1.5000
Kurtosis is 1.7754 and Skewness is 0.5820

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Figure I-4: Certify outlier test.

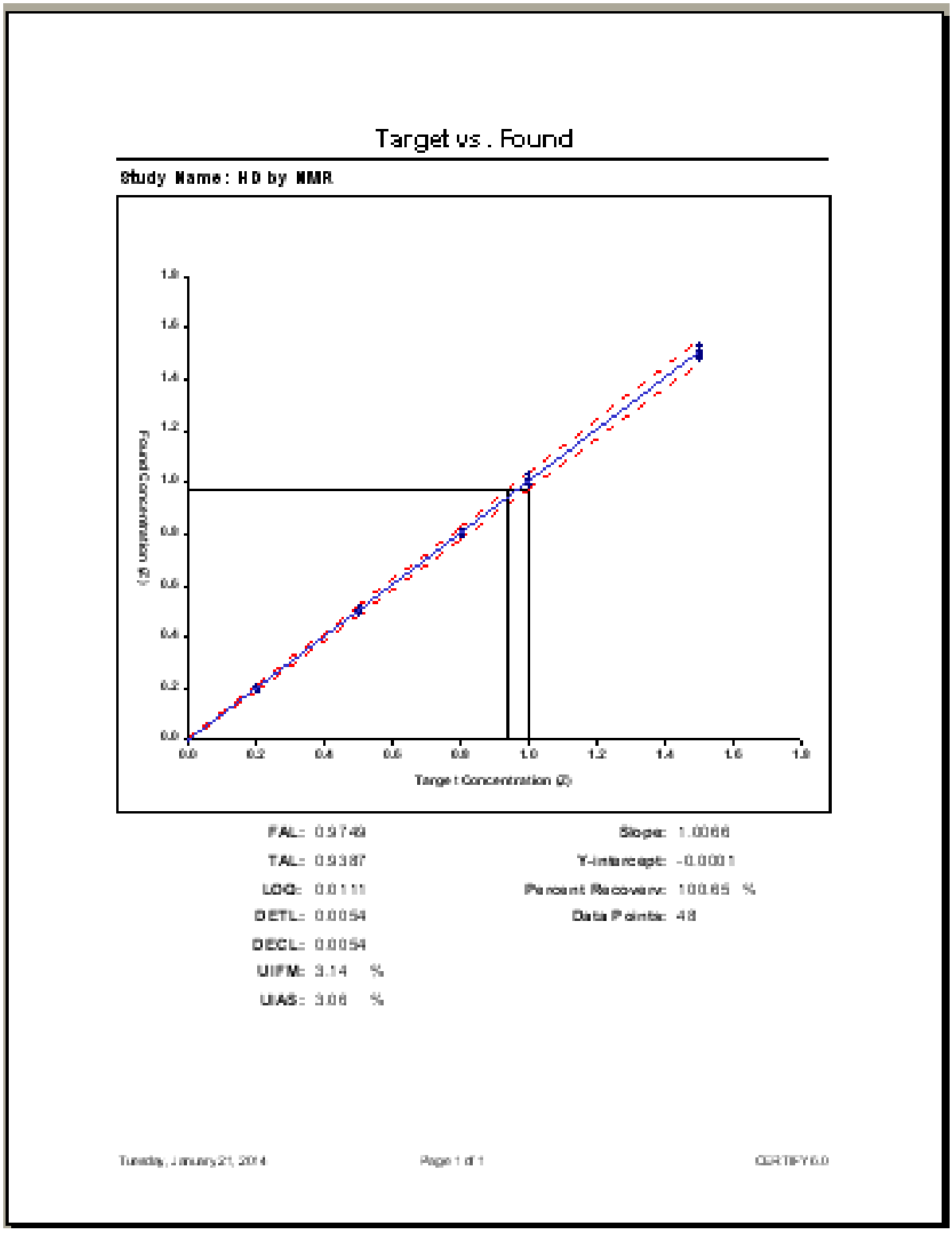

Figure I-5: Certify target Z vs. found Z plot screen.

APPENDIX II: Confirmation Data for the HD Method using ^{13}C NMR

II-1. APPROACH

The confirmation method for HD purity determination using ^{13}C spectra was not validated using a full Precision and Accuracy (P&A) test, due to limited time and amount of agent. However, several purity runs were done using the same lot of HD to determine the typical statistical uncertainty of the test. The statistical information is dependent on the type of NMR that was used (in this case, a JEOL ECS-400 400 MHz NMR), so it is not a general property of the analytical method, but it must be determined for each particular instrument.

The accuracy of the measurement depends on the signal to noise ratio of the spectrum, which depends on a number of factors: the amount of analyte that is present and the number of scans that are averaged. The accuracy also requires that the peak heights in the spectrum are proportional to the concentration in the sample, which can depend on the relaxation delay time.

The T_1 relaxation time is used to determine how long the relaxation delay time should be. For HD, the T_1 time for the ^{13}C peaks was determined using a typical inversion recovery experiment. A plot of the resulting recovery is shown in Figure II-1 for the 34 ppm peak of HD. This can be compared to the proton relaxation that is shown in Ref. 7.

The T_1 recoveries for the two HD peaks are 5.8 s (34 ppm) and 6.6 s (43 ppm). For the 1,1,1,2-tetrachloroethane internal standard, the T_1 recovery is longer, 8.7 s (59 ppm peak). The spectrum is shown in Figure II-2. (The recovery for the 96 ppm peak is much longer, 31 s, but this peak is not used for the purity determinations. The recovery of the $CDCl_3$ solvent peak is also long.) Based on the longest recovery, 8.7 s for the peaks which are used in the calculation, the relaxation time should be $10 \times T_1 = 87$ s. This relaxation time is 50% longer than the 60 s relaxation time that was suggested in the method discussion, and for optimum results the acquisition times should be 50% longer. The use of a relaxation time that is shorter than the optimum causes some distortion of the signal responses. The peak with a longer recovery time is not quite relaxed to the baseline, so the peak gets slightly saturated and has a lower response. This affects the purity result.

However, using a shorter relaxation time allows a trade-off of obtaining a better signal to noise ratio by signal averaging more repeat runs in the same amount of time.

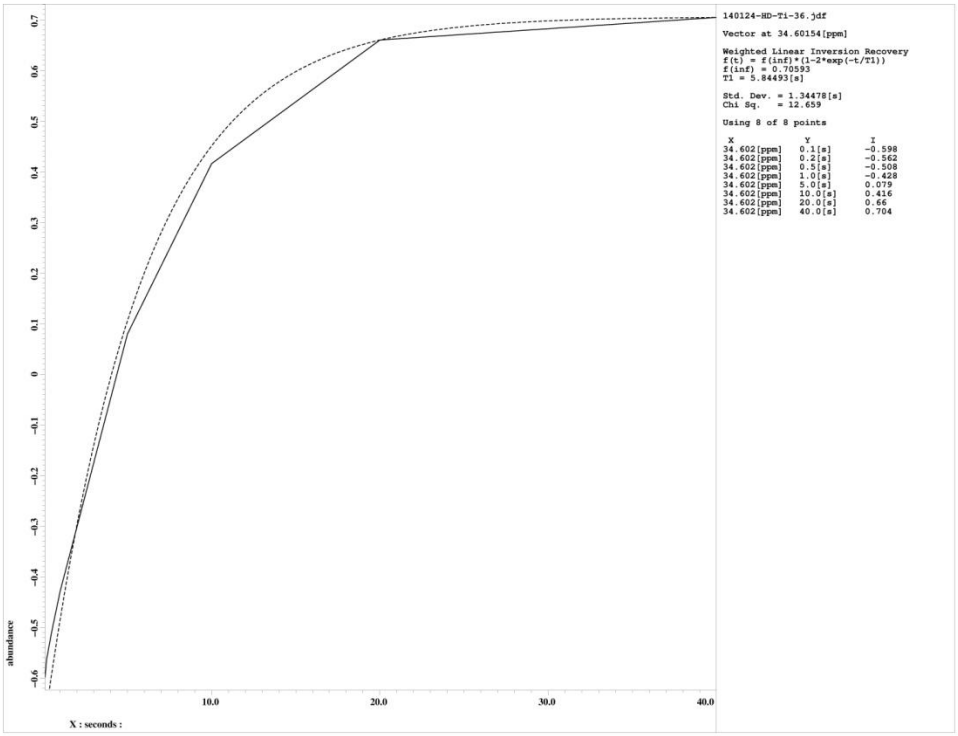

Figure II-1: Inversion recovery plot for the HD peak at 34 ppm in the ^{13}C spectrum. From the best fit curve using a nonlinear least squares fitting routine, the T_1 value is found to be 5.8 sec.

The spectrum shown in Figure 3 took 8.5 hr to acquire using 25 mg of HD, with 512 scans and a delay time of 60 s, giving a signal to noise ratio of 241. By increasing the amount of HD to 38 mg and decreasing the number of scans to 128, a signal to noise ratio of 79 was obtained with a 2 hr acquisition time. A longer acquisition time can give a more accurate result, but in order to perform 7 repetitions to get statistical information, a shorter runtime is often needed to minimize the instrument time.

One long acquisition was done on the same sample as was used for the proton purity to compare the purity determination. This run gave a purity result of 96.2 wt.%, calculating with the carbon peak from the methyl groups of the internal standard compound. If the purity is calculated using the phenyl (ring) carbon atoms, the purity result is 95.2 wt.%. The spectrum can also be run without the proton decoupler, which should decrease distortion of the carbon peak areas from the coupling to the protons, and this approach gave a purity result of 97.1 wt.%. However, this method gives lower signal to noise ratio, which was only 63 for an 8.5-hr run.

A set of 7 repetitions was done using the ^{13}C purity method to compare with 3 repetitions on the same sample with a proton purity method. The same lot of HD was used as was used for the results in Appendix I, but it was done over a year later on 26 Nov. 2013. The internal standard for this set was 1,1,1,2-tetrachloroethane, which was used so that both the HD and internal standard contained CH_2 carbons, avoiding enhancement of one compound from different numbers of H atoms on the carbon. A weight of 31.7 mg of HD was used.

The purity for HD using the proton method was 100.42 wt.%, with a standard deviation of 0.12, using only the 3.65 ppm triplet in the spectrum for the determination. The ^{13}C runs were 2 hr long, using 128 scans and a relaxation delay of 60 s, to give a signal to noise ratio of 79. A sample spectrum is shown in Figure II-2. The purity from the ^{13}C method was 96.34 wt.%, with a standard deviation of 6.7. The results of the two measurements are within one standard deviation, but the proton determination is higher than the ^{13}C determination. The reason that the proton result is systematically higher than the ^{13}C result is not known, but it may be due to the relaxation time being shorter than is optimum.

A study of the signal to noise ratio for ^{13}C spectra as a function of number of scans is given in Table II-1. It is expected from theoretical grounds that the signal to noise ratio should increase with the square root of the number of scans. The standard deviation of the purity determination is not the same as the signal to noise ratio, although they should be roughly related to each other. From this data, it is necessary to use about 200 scans to get a 100:1 signal to noise ratio, corresponding to about a 1% accuracy.

Table II-1: Data showing the relationship of signal to noise ratio to the number of scans for ^{13}C spectra.

Number of scans	√ns	S/N for 34 ppm peak	Run time (h)
16	4	24	0.5
64	8	50	1.75
144	12	71	3.8
196	14	90	5.1
400	20	127	10.3

A reason for using ^{13}C spectra rather than proton spectra is for the higher spectral resolution. In particular, the compound dithiane is present in the HD sample at a concentration of about 1 wt.%. This peak is resolved from the HD in the ^{13}C spectrum, and it is labeled in Figure 3. But the proton spectrum does not have enough resolution to separate the 2.9 ppm

triplet peak of HD from the dithiane peak. This example illustrates the need to trade off the resolution of the ^{13}C spectrum for the sensitivity of the proton spectrum.

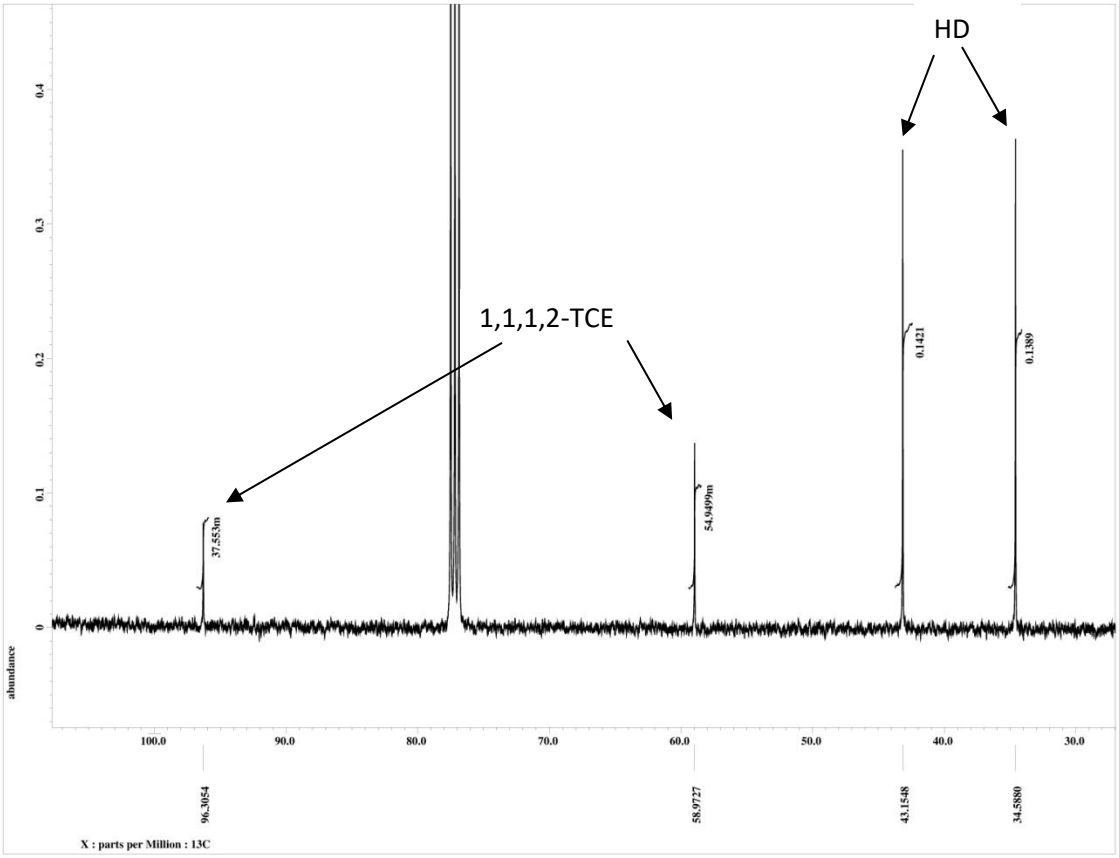

Figure II-2: ^{13}C NMR spectrum of HD with an internal standard of 1,1,1,2-tetrachloroethane. Integrals and peak labels are shown.

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